Original Research Article

Circulating level of platelet activation biomarker -Platelet factor 4, in sickle cell anaemia patients in Edo State, Nigeria

ABSTRACT

Aim: To assess the circulating level of platelet activation biomarker platelet factor 4 in sickle cell anaemia patients attending a sickle cell foundation in Edo State, Nigeria.
Study design: Case-controlled observational Study
Place and Duration of Study: Sickle Cell Foundation Benin City, Edo State and Afe Babalola University, Multisystem Hospital, Ado Ekiti, Ekiti State, Nigeria, between April 2020 - September 2020.
Methodology: Forty (40) Haemoglobin (Hb) SS genotype subjects diagnosed by haemoglobin electrophoresis and aged 10 to 40 years were selected for the study. Twenty (20) Hb AA and 20 Hb AS apparently healthy subjects were used as control. The Hb SS subjects in their steady and crisis states who met the eligibility criteria were used as test subjects and verbal and written consents were obtained from all participants. Venous blood was collected for this study, in which 5ml of the subjects' venous blood was collected with a syringe, 3ml was put into an Ethylene Diamene Tetra acetic acid container for the evaluation of Platelet count. Platelet indices and the Haemoglobin Genotyping, while 2ml was put into a plain container for the serological assay (Platelet Factor 4 assay), using ELISA method. The platelet count was estimated manually using an improved Neubauer counting chamber and 1% ammonium oxalate, the Haemoglobin Genotype was evaluated manually using an electrophoresis tank and Tris buffer, Platelet indices was evaluated using an Hematology Auto Analyzer. Statistical Package for Social Sciences (SPSS) version 23 was used for statistical analysis and p values less than *0.05* were considered statistically significant.
Results: The results showed that the mean serum Platelet Factor 4(PF-4) was significantly reduced in the Sickle Cell Disease (SCD) crisis ($p<0.05$, respectively) and steady groups compared to the control group ($p<0.05$, respectively).
Conclusion: This study suggests that there is a significant increase in the platelet count in SCD crisis, and there is an increase in platelet count with increasing severity of the disease. Circulating PF-4 concentrations was also found to increase in Hb-AS compared to the SCD.

Keywords: Platelet activation, Platelet factor 4, sickle cell anaemia.

1. INTRODUCTION

Sickle cell anemia is the most common hemoglobinopathy and the most severe form results from homozygous inheritance which occurs due to a point mutation that results in the replacement of adenine by thymine (GAG → GTG) in the sixth codon of the *β*-globin gene (*β*S), specifically valine replacing glutamic acid at the sixth position of the polypeptide chain thereby yielding an abnormal form of hemoglobin (HbS) [1]. An estimated 300,000 children are born with a severe form of sickle cell anaemia condition(homozygotes) worldwide each year, mainly in the sub-Saharan Africa, Middle East, and India. Migration patterns led to the distribution of the sickle cell gene to non-endemic areas of malaria, such as some parts of

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Europe and the USA. In Brazil, in 2016, 1,071 newborn babies had sickle cell disease (SCD) and >60,000 were heterozygous for the β^S allele. There are an estimated 4.8 million individuals with SCD in the whole country [2].

Sickle cell anemia is associated with a chronic pro inflammatory state characterized by an elevated leukocyte count, mortality from severe recurrent infections, and subsequent vaso-occlusive complications such as leukocyte adhesion to the endothelium and increased plasma levels of inflammatory cytokines. The main events of the sickle cell anaemia pathophysiology are hemolysis and vaso-occlusive painful crises due to the changes and alterations that occur in the structure of the haemoglobin which is necessary for sickle cell anaemia to occur. This process results in an alteration of the normal lipid bi-layer and erythrocyte membrane proteins, which then results in changes to the erythrocyte shape and physical properties of the erythrocyte. These changes eventually result in hemolytic anaemia, shortened red cell survival, unregulated interactions with other blood cells, red cell precursor deaths, blockage of blood flow, which may lead to repeated hypoxia-reperfusion processes further damaging any other organs [3].

The platelet is a small, anucleated cell originally derived from the hematopoietic lineage called the megakaryocyte. Platelets production from the megakaryocytes is a systematic and regulated process occurring either in the bone marrow or, in the lungs as reported more recently. The primary role of the platelet in circulation is to help maintain primary hemostasis and leakage within the vessel and in accomplishing this goal; the platelet flows through the vessel in close proximity to the vessel wall due to the biophysical nature of the blood constituents and shear forces within the vessel [4]. This close proximity to the vessel wall allows for a quick response when a vascular damage or injury occurs and this response is typically thought to occur in several stages starting with adhesion to the subendothelial extracellular matrix through initial interaction of the matrix with specific receptors on the platelet [4]. These platelet specific receptors include the GP1b/V/IX complex binding to Von Willebrand factor as well as GPVI and αIIβ1 receptors on the platelet surface binding to the collagen component of the extracellular matrix [5]. Although platelet adhesion, aggregation, and release have been intensively investigated, little is known about how products of the platelet release reaction exert their inflammatory response.

Platelet factor 4 is a chemotactic protein for polymorphonuclear leukocytes and monocytes which are released from the alpha granules when platelets adhere at the site of blood-vessel injury. It is a relatively small, heat-stable protein which upon release binds to a carrier proteoglycan molecule from which it is displaced by heparin [6,7]. The chemokine family can be subdivided into CXC and CC chemokine ligands (CXCL, CCL), the CXC chemokine platelet factor-4 (PF-4/CXCL4) is a major constituent of platelet α-granules released in high amounts upon platelet activation [8,9]. The biosynthesis of Platelet Factor 4 is almost exclusively limited to megakaryocytes from which mature circulating platelets are derived and account for ~2% of the total α- granular content on a molar basis in platelets while low concentrations of it have also been recently reported as synthesized in activated human monocytes. Unlike the related chemokines interleukin (IL)-8 and neutrophil activating peptide (NAP)-2, Platelet Factor 4 does not possess significant chemotactic activity for neutrophils or monocytes [10]. It exerts its neutrophil activation ability, initiate exocytosis and enhance protein matrices or endothelial adhesion by interacting with a co-stimulus such as tumor necrosis factor (TNF)-α [11]. Additionally, the platelet factor 4 possesses an array of effects on circulating monocytes, preventing apoptosis and facilitating macrophage differentiation during the inflammatory process [12]. It has also been associated with augmenting monocyte phagocytosis and subsequent respiratory burst and to induce cytokine secretion [13]. The aim of this study was to assess the circulating level of platelet activation biomarker platelet factor 4 in sickle cell anaemia patients attending a sickle cell foundation in Edo State, Nigeria.

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2. MATERIALS AND METHODS

2.1 Study Area

This study was conducted at the Sickle cell foundation located in the Government reserved area of Benin-City, Edo State. The sickle cell foundation is a secondary health institution tasked with providing services in improving the standard of healthcare of individuals with sickle cell disorder, genetic counselling, prenatal diagnosis, newborn screening, leg ulceration treatment & transcranial doppler ultrasound. Benin is the capital of Edo state, which is an inland state in Nigeria, and it is located at the central part of Nigeria.

2.2 Study Population

The study population consisted of 80 subjects who were categorised into four (4) groups, which includes group A having 20 subjects of HbAA as healthy control, group B having 20 subjects of HbAS group C with 20 subjects of HbSS at steady state and group D having 20 HbSS subjects in vaso-occlusive crisis state who were age, sex and socioeconomic standard matched were used for this study. The other study participants were recruited from the General Out Patient Department (GOPD) of Central Hospital Benin City and Afe Babalola University Multi-System Hospital, Ado-Ekiti, Ekiti State.

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2.3 Study Design

~~The study is a case-control study involving participants from the Sickle Cell Foundation in Benin City, Edo state and laboratory analyses were carried out in the Afe Babalola University Ado-Ekiti, Ekiti State; Nigeria and at a research facility in Ilorin, Kwara State respectively. Forty (40) HbSS subjects diagnosed by haemoglobin electrophoresis aged 10 to 40 years were selected for the study, twenty (20) Hb AA and 20 Hb AS apparently healthy subjects were used as control. The Hb SS subjects in their steady and crisis states who met the eligibility criteria were used as test subjects and verbal and written consents were obtained from all participants.~~

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2.3.1 Experimental Grouping

This experiment had 4 groups;
Group A- HbAA subjects (Healthy control)
Group B- HbAS subjects
Group C-HbSS subjects (Steady state)
Group D- HbSS subjects (Vaso-occlusive state).

2.4 Sample Size Determination

The minimum sample size for this study was calculated using:

$$N = \frac{Z^2 \times P(1-P)}{d^2} \quad [14]$$

Where N= minimum sample size,
Z= confidence interval at 95%,
P= the prevalence of Sickle cell disease in Benin City, Edo State at 2.39%= 0.0239 (Nwogoh *et al*., 2012).
d= desired level of significance (0.05)

$$N = \frac{1.96^2 \times 0.0239 \times (1-0.0239)}{0.05^2}$$

$$N = \frac{3.8416 \times 0.0239 \times 0.9761}{0.0025}$$

$$N = 35.85$$

Sample size (n) 35.85 is the minimum number of participants that can be recruited for the study; nevertheless, 20 participants were enlisted in the various groups in order to reduce possible errors to a bare minimum.

2.5 Eligibility Criteria

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2.5.1 Inclusion Criteria:

- HbSS subjects with established diagnosis confirmed through haemoglobin electrophoresis.
- Healthy individuals without any form of illness.
- HbSS patients not on antiplatelet therapy.
- Confirmed HbSS subjects, male and female within the 10-45 years age bracket.
- HbSS patients without a history and case of thrombocytopenia.

2.5.2 Exclusion Criteria:

~~Respondents who are;~~

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- HbSS patients taking antiplatelet therapy.
- Pregnant sickle cell patients.
- Subjects with any form of illness or disease.
- HbSS subjects outside the 10-45 years age bracket.
- HbSS subjects who are taking NSAIDs.

2.6 Sample Collection, Preparation and Analysis

2.6.1 Sample Collection

Venous blood was collected for this study, in which 5ml of the subjects' venous blood was collected with a syringe, 3ml was put into an Ethylene Diamene Tetra acetic acid container for the evaluation of the required Hematological Parameters, while 2ml was put into a plain container for the serological assay.

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2.6.2 Sample Preparation

Five (5) ml of venous blood was obtained from each subject, with three (3) ml of blood each carefully dispensed into ethylenediaminetetra-acetic acid (EDTA) bottles for haematological analysis using conventional methods and the remaining two (2) ml dispensed into plain bottle for serological assay. The serum was gotten by centrifuging the samples in the plain bottle at 3000rpm for 15minutes and extracted into cryotubes for storage at -20^{o}C until the samples were pooled for cytokine analysis.

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2.6.3 Sample Analysis

2.6.3.1 Haemoglobin Genotyping using Haemoglobin Electrophoresis [15].

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Haemoglobin electrophoresis was carried out using the cellulose acetate paper and Tris borate EDTA buffer as the stationary and mobile phases respectively.

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2.6.3.2 Estimation of Platelet Count [16]

Platelet count was determined using the haemocytometeric method.

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2.6.3.3 Estimation of Platelet Indices [16]

Platelet indices, plateletcrit (PCT), mean platelet volume (MPV), and platelet distribution width (PDW), were determined using a Haematology auto-analyzer.

2.6.3.4 Assessment of Platelet factor 4 using ELISA method [17].

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The level of platelet factor 4 was assessed using the sandwich ELISA method which quantifies antigens between two layers of antibodies (that is, capture and detection antibody).

2.7 Statistical Analysis

All statistical calculations were performed with the Statistical Package for Social Sciences (SPSS) version 23 for Windows 7 program and p values less than 0.05 were considered statistically significant. The correlation of results obtained was represented in tables and figures.

3. RESULTS AND DISCUSSION

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Before starting the discussion , describe the sigificant results very brifly in text, refering to the relevat tables

Table 1. Mean age distribution and sex of study subjects

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Age	Control	AS	Crisis	Steady	
Age (years) (mean ± SD)	22.68 ± 5.14	24.27 ± 3.76	21.92 ± 3.95	22.67 ± 6.39	
Gender					
Male (%)	14 (36.8%)	15 (40.5%)	15 (40.5%)	5 (33.3%)	
Female (%)	24 (63.2%)	22 (59.5%)	22 (59.5%)	10 (66.7%)	0.878

Table 2. Comparison of the mean values of platelet and platelet indices of sickle cell anaemia patients with the Control group

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Parameter	Control ± SD (n=28)	AS ± SD (n=22)	SCD Crisis ± SD (n=14)	SCD Steady ± SD (n=16)	P-
Platelets ($x10^3$/µL)	205416.7 ± 8214.59	202583.3±16999.8	507285.7* ± 74187.65 [abc]	307000.0±27833.3	<0.001
MPV ($µm^3$)	8.0 ± 0.14	8.2 ± 0.27	8.3 ± 0.24	8.35±0.18	0.45
PDW (%)	14.89±0.34	16.38±1.66	15.10±0.74	14.44±0.58	0.021

a - significantly different from control; b - significantly different from AS, c- significantly different from Steady.

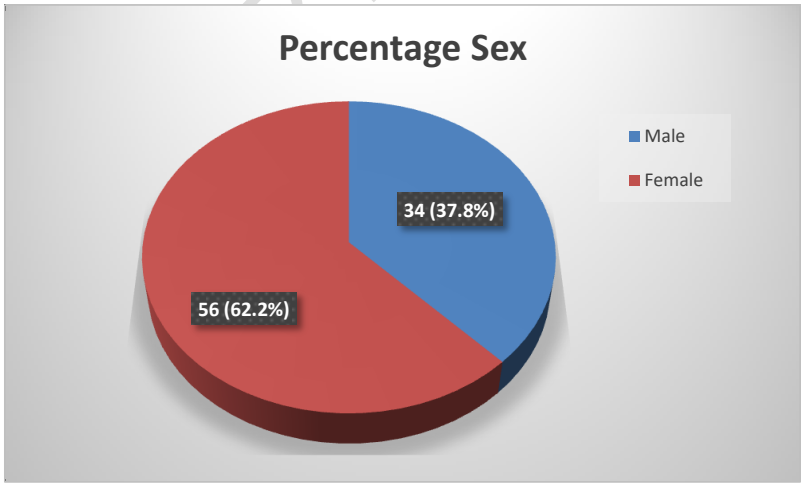

Figure 1: Gender profile of the study population

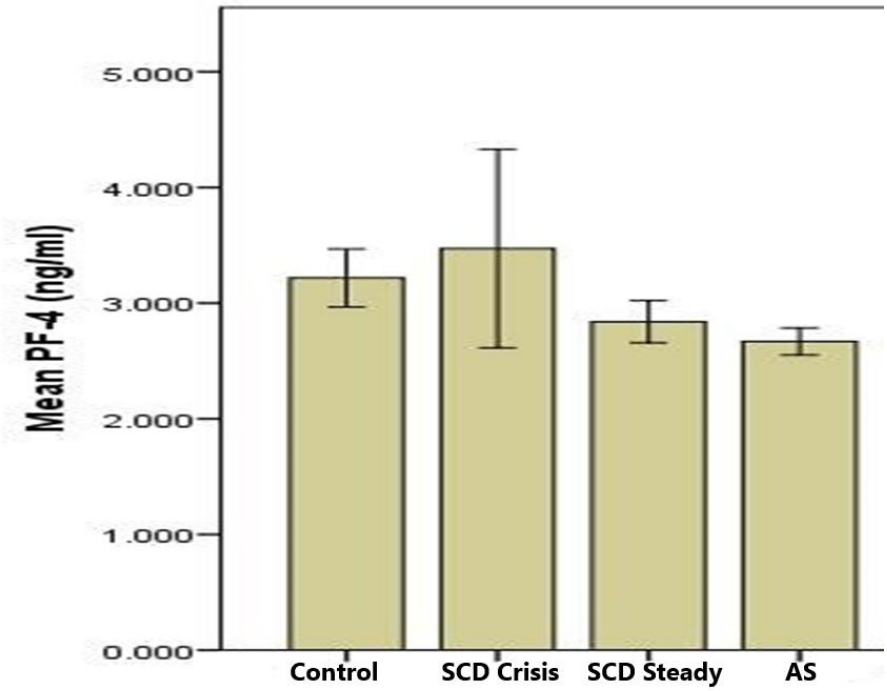

Figure 2: Graphical representation of serum platelet factor 4 (PF-4) profile of sickle cell anaemia subjects in comparison with control group.

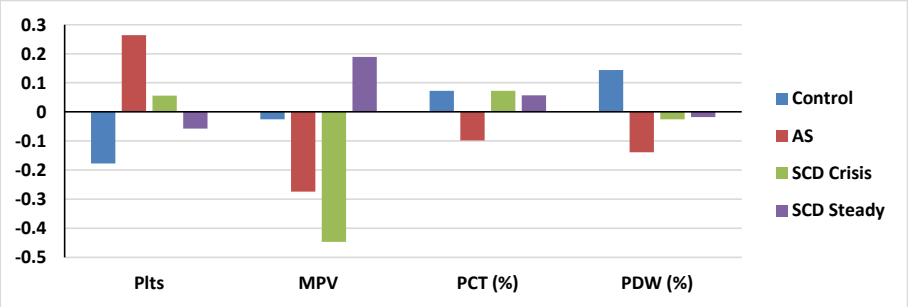

Figure 3: Correlation between platelet factor 4 (PF-4), Platelet count, MPV, PCT and PDW among the SCD groups and control group.

Table 3: Crisis and steady state of SCD

Group	Variable	Control		SCD Crisis		SCD Steady		AS	
		r	P	r	p	R	p	r	p
PF-4 (ng/ml)	**Plts ($x10^3$/µl)**	-0.177	0.228	0.264	0.407	0.056	0.440	-0.057	0.833
	MPV	-0.026	0.861	-0.274	0.381	***0.447**	**0.019**	0.189	0.483
	PCT (%)	0.073	0.622	-0.098	0.761	0.072	0.818	0.057	0.833
	PDW (%)	0.144	0.328	-0.139	0.668	***0.026**	**0.029**	-0.018	0.946

***Correlation is significant at the 0.01 level (2-tailed)**

Sickle cell disease (SCD) is characterized by the presence of sickle hemoglobin, which has the unique property of polymerizing when deoxygenated. The pathophysiology of acute and chronic clinical manifestations of SCD have shown the central role of dense, dehydrated red cells in acute and chronic clinical manifestations of this pathology. SCD is characterized by a hypercoagulable state that contributes to the vaso-occlusive events in microcirculation, leading to acute and chronic sickle cell– related organ damage [18]. This research assessed platelet count, platelet indices and platelet factor 4 in known sickle cell anaemia subjects and control subjects.

Discussion In this study, a significant increase in the platelet count of the SCD Crisis group was observed when compared with the Control, AS and SCD steady groups. The clinical picture of sickle cell disease (SCD) is dominated by complications arising from vaso-occlusive crisis (VOC). VOC is precipitated by complex interactions between sickled erythrocytes, endothelial cells, leukocytes, platelets, and plasma proteins. The findings of this study agree with Durjoy et al. [19]. Increased platelet count (thrombocytosis) in SCD is associated with underlying inflammation which result ~~to the~~ increased platelet count.

In this study, there was no significant difference in the MPV and PDW of the SCD crisis group when compared to the Control group. The mean platelet counts however increased with increasing severity of the sickle cell anaemia. No significant difference was observed in the mean platelet count, MPV and PDW of the AS and SCD steady groups when compared with the control group.

In this study the Hb-AS group had significantly increased circulating PF-4 concentrations compared to the SCD groups. The mean serum PF-4 was however significantly reduced in the SCD crisis and steady groups compared to the control group. However, no significant difference was observed in the circulating PF-4 level between the SCD groups when compared together. PF-4 was generally increased in patients with sickle cell anaemia than those without sickle cell anaemia. The findings of this study agree with Tomer *et al.*, 2001 [20]. An increase in activation factors and plasma soluble factors as Platelet consumption increases during SCD pain episodes, resulting in transient thrombocytopenia and subsequent rebound thrombocytopenia, as well as elevated plasma levels of secreted platelet products as well as PF-4. may be associated with sickle cell disease.

In this study there is a positive correlation of PF-4 with MPV and PDW in the SCD crisis group; with PF-4 increasing as the MPV and PDW increase in the SCD crisis group. However, no significant correlation observed between the platelet count, PCT, and serum

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PF-4 in the SCD crisis group. There was no significant correlation between the plasma concentration of platelet factor 4 and platelet indices in the AS, SCA steady and control groups. This agrees with Amin et al. [21], as MPV and PDW increase during platelet activation.

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4. CONCLUSION

This study suggests that there is a significant increase in the platelet count in SCD crisis subjects when compared to the control and SCD steady state groups, and it also indicates that the platelet count, increases with increasing severity of the disease. Furthermore, the Hb-AS group had significantly increased circulating PF-4 concentrations compared to the SCD groups.

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What is the implication of this fniding?
How does this conclusiion contribute to diagnosis, treatment ad prognosis of sickle cell crisis?
Briefly incarpoarte the answers to these questions in the title, aims,conclusion and disicussion.

CONSENT

Informed consent was obtained from various participants after vividly explaining the purpose of the study and assuring them of the confidentiality of their results.

ETHICAL APPROVAL

The ethical approval for the experiment was acquired from the Chairman of the Ethical Committee, Edo State Ministry of Health, Benin City, Edo State.

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